0022-202X/84/8302-0153$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 83:153–156, 1984

Vol. 83, No. 3
*Printed in U.S.A.*

REPORTS

# $\Delta^5$-3$\beta$-Hydroxysteroid Dehydrogenase $\Delta^{4-5}$-Isomerase Activity and Metabolism of Dehydroepiandrosterone in Rat Preputial Gland

MARTY E. SAWAYA, PH.D., WALTER VOIGT, PH.D., AND SUNG L. HSIA, PH.D.

*Departments of Biochemistry, Pathology, and Dermatology and Cutaneous Surgery, University of Miami School of Medicine, Miami, Florida, U.S.A.*

**After intracardial injection of [1,2-$^3$H]dehydroepiandrosterone ([$^3$H]DHA) into female rats, [$^3$H]DHA was found to accumulate and was metabolized in the preputial gland, but not in the diaphragm. The identified metabolites of [$^3$H]DHA in the preputial gland were $\Delta^4$-androstenedione-3$\alpha$,17$\beta$-diol. Cells were isolated from the preputial gland after treatment with trypsin and collagenase III, and centrifugation in Ficoll gradients. Activity of the enzyme complex $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase $\Delta^{4-5}$-isomerase (3$\beta$-HSD) responsible for transforming DHA into $\Delta^4$-androstenedione was found mainly in the 105,000 *g* pellet (microsomal fraction) of homogenates of the isolated cells. It used preferentially NAD over NADP as a coenzyme, with a pH optimum at 8.5. The apparent $K_m$ for DHA was 5.5 × $10^{-5}$ M, and the $V_{max}$ was 1.72 nmol/min/mg microsomal protein. These findings indicate that DHA is preferentially taken up by the preputial gland where it undergoes metabolism to form more potent androgens, and suggest that DHA may have important androgenic influence on the preputial gland.**

The influence of dehydroepiandrosterone (DHA) on sebaceous activity has been implicated in a number of studies. In patients with cystic acne, a significant correlation has been found between serum levels of dehydroepiandrosterone sulfate (DHAS) and the number of lesions [1]. Increased sebum production has been reported after the administration of DHA to adult males whose sebaceous secretion had been suppressed by ethynyl estradiol [2,3], and of DHAS to hypogonadal males [4]. The influence of DHA and DHAS on sebaceous activity is thought to be mediated by their conversion to more potent androgenic steroids, $\Delta^4$-androstenedione, testosterone, and dihydrotestosterone (DHT) in the skin [5–7]. A significant correlation has been reported between the activity of $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase $\Delta^{4-5}$-isomerase (3$\beta$-HSD) in human sebaceous glands and their secretory activity [8]. 3$\beta$-HSD is the enzyme complex that converts DHA to $\Delta^4$-androstenedione, which is subsequently transformed into testosterone and DHT in the skin [9].

It is a common clinical observation that acne lesions often flare up or exacerbate when the patient is under stressful conditions, possibly linked to the excessive secretion of adrenal steroids in response to stress. Since DHA is the major adrenal androgen, its action on sebaceous glands, especially, merits investigation.

Because of the practical limitations in obtaining human sebaceous cells for detailed biochemical studies, we have used the rat preputial gland to investigate the androgenic action of DHA. Growth of the gland has been shown to respond to the stimulation of androgenic, but not estrogenic or adrenocortical steroids [10]. A pair of the glands is situated in the prepuce of the rat of either sex and weigh 175–200 mg in an average 200-g rat, being slightly larger in the male animal.

In a previous study [11], we treated the gland tissue with trypsin and collagenase III, according to the method of Potter et al [12], and separated the cells by centrifugation into 5 bands according to lipid contents. We identified $\Delta^4$-androstenedione, testosterone, and DHT as the major metabolites of DHA in the isolated cells, and also observed a significant reduction of DHT content in the gland after adrenalectomy of male and female rats. These findings support the view that the androgenicity of DHA is mediated by conversion into DHT in the gland and that the adrenal cortex is an important source of androgen for the gland.

In this paper, we report our continued study of androgenic action of DHA in rat preputial gland, and present data on the characterization of 3$\beta$-HSD, the preferential uptake of [1,2-$^3$H]DHA by the gland, and subsequent metabolism to form $\Delta^4$-androstenedione, testosterone, DHT, and androstane-3$\alpha$,17$\beta$-diol.

## MATERIALS AND METHODS

### *Chemicals*

[1,2-$^3$H]DHA (40 Ci/mmol), [1,2-$^3$H]androstenedione (40 Ci/mmol), [1,2-$^3$H]testosterone (40 Ci/mmol), and [1,2-$^3$H]DHT (40 Ci/mmol) were purchased from New England Nuclear. Each steroid was checked by thin-layer chromatography to have greater than 95% radiopurity. The benzene solutions as purchased, were evaporated under nitrogen and the radioactive steroids were dissolved in ethanol to contain approximately $10^6$ dpm in 0.01 ml, and the solutions were stored at 10°C.

Reagent-grade solvents, acids, bases, and salts were from Mallinckrodt. Silica gel H for the preparation of thin-layer chromatography plates was a product of Merck A.G. Co. Coenzymes, EDTA, Tris, bovine serum albumin, catalase, and other chemicals were from Sigma Chemical Co. Reference steroids were purchased from Steraloids, Inc.

### *Animals*

Female Sprague-Dawley rats, 10–12 weeks old, weiging 225–300 g were housed under standard conditions of controlled temperature, humidity, light and dark cycles, and allowed access to commercial rat chow pellets and drinking water ad libitum.

### *In Vivo Uptake and Metabolism of DHA*

Each rat, under anesthesia with ethyl ether, was injected intracardially with 5 $\mu$Ci of [1,2-$^3$H]DHA in 0.1 ml of Tris buffer saline solution,

Manuscript received Feburary 6, 1984; accepted for publication April 6, 1984.

Supported in part by a grant from the Dermatology Foundation of Miami.

Reprint requests to: S. H. Hsia, Ph.D., Department of Dermatology and Cutaneous Surgery—R117, University of Miami School of Medicine, P.O. Box 016960, Miami, Florida 33101.

Abbreviations:
DHA: dehydroepiandrosterone
DHAS: dehydroepiandrosterone sulfate
DHT: dihydrotestosterone
[$^3$H]DHA: [1,2-$^3$H]dehydroepiandrosterone
3$\beta$-HSD: $\Delta^5$-3$\beta$-phydroxysteroid dehydrogenase $\Delta^{4-5}$-isomerase

using a tuberculin syringe with a 25-gauge needle. At 30-, 45-, and 60-min intervals after the injection, the animals were killed, 3 in a group, by a blow behind the head. The diaphragm muscle and the preputial gland were excised for comparison of DHA uptake and metabolism.

The excised tissues were dissected, weighed, and immediately placed in glass vials containing methanol and kept on ice. The tissues were minced and extracted with methanol:chloroform (2:1, v/v), and then homogenized with a Ten Broeck all-glass homogenizer in the same solvent mixture. The extracts were combined and evaporated to dryness under $N_2$ gas. Carrier steroids (50 $\mu$g each of testosterone, DHT, $\Delta^4$-androstenedione, androstanedione, androstane-3$\alpha$,17$\beta$-diol, $\Delta^5$-androstene-3$\beta$-17$\beta$-diol, and DHA) were added, and the mixture was dissolved with ethanol and applied to thin-layer plates for chromatographic separation as previously described [11]. The carrier steroids were visualized by exposure to iodine vapor, and the plates scraped in 1-cm sections and assayed for radioactivity in a scintillation counter to locate the areas of radioactivity.

*Assay for 3$\beta$-HSD Activity*

The excised preputial glands were treated with collagenase III and trypsin, according to the method of Potter et al [12], with minor modifications as described in our previous communication [11]. Five bands of cells were isolated after centrifugation in discontinuous gradients of 0, 5, 10, 15, and 20% Ficoll in Hanks' solution. The cytologic characteristics with regard to size, density, and protein contents of the cells in the 5 bands, as well as their enzymic activities for steroid metabolism, have been described previously [11]. The cells harvested at the interfaces of the 10 and 15%, and the 15 and 20% Ficoll solutions (bands IV and V), were combined and homogenized with a Ten Broeck all-glass tissue homogenizer in 0.1 M citrate-phosphate buffer, pH 7.4. The cellular homogenate was filtered through 4 layers of cheesecloth and then separated into nuclear, mitochondrial, microsomal, and cytosolic fractions by sequential centrifugation at 800 *g*, 10,000 *g*, and 100,000 *g* in 0.1 M citrate-phosphate buffer, pH 7.4. The pellets were resuspended in 0.1 M citrate-phosphate buffer and resedimented by centrifugation.

For assay of 3$\beta$-HSD activity, [$^3$H]DHA ($10^6$ dpm) and 100 nmol of nonradioactive DHA in 0.01 ml ethanol were added to the subcellular fractions which contained the equivalent of 1 g of tissue in a final volume of 1 ml of 0.1 M citrate-phosphate buffer containing 5 $\mu$mol of NAD or other coenzymes as specified. Unless otherwise indicated, the pH was 8.5, and the mixture was incubated in a Dubanoff metabolic shaker at 37°C for 20 min, when the formation of testosterone was undetectable. The incubation was terminated by immersing the incubation tube in a dry ice-acetone bath, and the content was lyophilized. Approximately 100 $\mu$g of each carrier steroid, testosterone, DHT, $\Delta^4$-androstenedione, androstanedione, androstane-3$\alpha$,17$\beta$-diol, $\Delta^5$-androstene-3$\beta$,17$\beta$-diol, and DHA were added and the mixture was extracted repeatedly with ethanol and applied to thin-layer plates for chromatographic separation. The recovery of $^3$H by this procedure was >95%. The radioactivity in the band containing $\Delta^4$-androstenedione was used to calculate 3$\beta$-HSD activity.

## RESULTS

*In Vivo Uptake and Metabolism of DHA by the Preputial Gland*

Fig 1 shows the comparison of the accumulation of $^3$H in the preputial gland and the diaphragm of female rats after injection of 5 $\mu$Ci of [$^3$H]DHA. The diaphragm was selected for the comparison because it is not an androgen-dependent tissue. The content of [$^3$H]DHA (measured in $10^3$ cpm/100 mg tissue) in the diaphragm was 3 times that in the preputial gland 30 min after the administration of [$^3$H]DHA, but it quickly declined, while that in the preputial gland continued to rise. At 60 min after the injection, the concentration of $^3$H in the preputial gland already exceeded that in the diaphragm, and the trend continued.

The $^3$H-labeled steroids were extracted from the tissues and analyzed by thin-layer chromatography. Fig 2 shows that [$^3$H]DHA was extensively metabolized in the preputial gland to form $\Delta^4$-androstenedione, testosterone, DHT, and androstane-3$\alpha$,17$\beta$-diol, while the [$^3$H]DHA in the diaphragm remained unmetabolized as no metabolite could be detected by thin-layer chromatography. The amount of $^3$H in DHA and each of the metabolites in the preputial gland increased with time, while

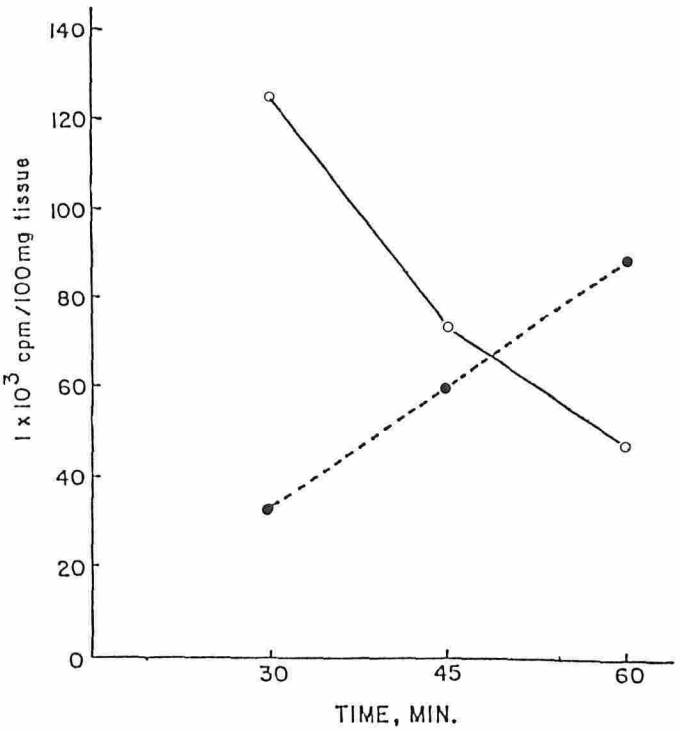


FIG 1. Uptake of [$^3$H]DHA in rat preputial gland (●– –●) and diaphragm (○———○) after intracardial injection of [$^3$H]DHA. The data represent the average results from 3 animals.

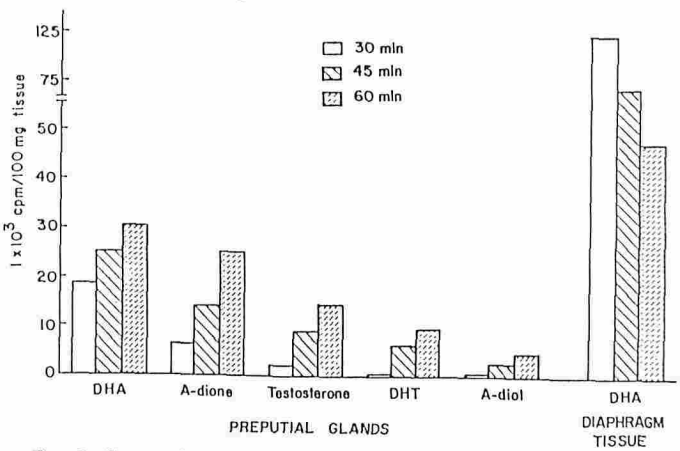


FIG 2. Comparison of retention and metabolism of DHA in rat preputial gland and diaphragm in vivo. The height of each *bar* represents the average of results obtained from 3 animals.

$^3$H in DHA in the diaphragm continued to decline. At 60 min, more than half of the [$^3$H]DHA in the preputial gland was transformed into the metabolites indicated above. These data demonstrate that [$^3$H]DHA is preferentially taken up by the preputial gland after intracardial injection, while the diaphragm, in spite of the initial high content of [$^3$H]DHA, does not accumulate [$^3$H]DHA. The data also show that the [$^3$H]DHA is readily metabolized by the preputial gland, but not by the diaphragm.

*Subcellular Localization and Some Characteristics of 3$\beta$-HSD*

The distribution of 3$\beta$-HSD activity in subcellular fractions of cells from bands 4 and 5 isolated from the preputial gland is shown in Table I. Most of the 3$\beta$-HSD activity was found in the microsomal fraction (105,000 *g* pellet). The cofactor requirement for 3$\beta$-HSD activity as assayed with the microsomal fraction, is shown in Table II. The preferred coenzyme was NAD. The pH dependence of the enzymic activity is shown in Fig 3; the peak of activity was around pH 8.5.

TABLE I. *Distribution of 3β-HSD activity in subcellular fractions of isolated cells from rat preputial gland*

| | Androstenedione produced | |
|---|---|---|
| | nmol | nmol/h/mg protein |
| Nuclear pellet (800 *g*) | 20 | 4.19 |
| Mitochondrial pellet (10,000 *g*) | 24.5 | 6.85 |
| Microsomal pellet (105,000 *g*) | 49 | 25.65 |
| High speed supernatant | 7 | 4.24 |

Cell bands IV and V, obtained after Ficoll gradient centrifugation, were combined and homogenized with Ten Broeck tissue grinder in 0.1 M citrate-phosphate buffer, pH 7.4. The pellets were resuspended and washed in the same buffer. Each fraction (in an amount equivalent to 1 g of tissue) was incubated with [$H^3$]DHA ($10^6$ dpm), and 100 nmol of nonradioactive DHA added to 20 μl of ethanol, for 30 min at 37°C in 1 ml of citrate-phosphate buffer, pH 8.5, containing 5 μmol of NAD.

TABLE II. *Cofactor requirement of 3β-HSD*

| Addition | DHA converted to androstenedione (nmol) |
|---|---|
| NAD | 48.7 |
| NADH | 19.3 |
| NADP | 26.4 |
| NADPH | 10.8 |
| None | 0 |

The incubation mixture contained 5 μmol of the indicated pyridine nucleotide with microsomes (2 mg of protein), [$H^3$]DHA ($10^6$ dpm; 10 pmol) and 100 nmol of nonradioactive DHA in 0.1 M citrate-phosphate buffer, pH 7.4. Incubation was performed at 37°C, for 30 min.

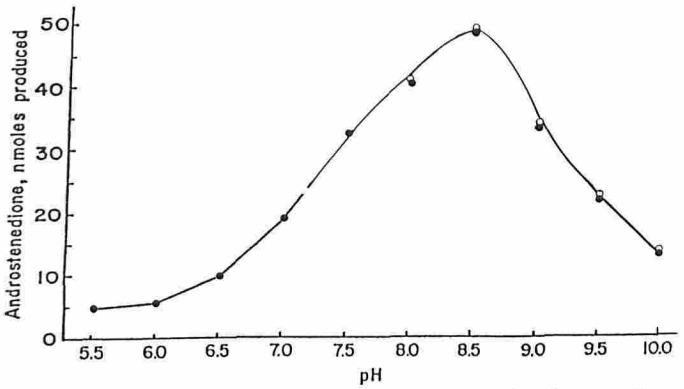


FIG 3. pH dependence of conversion of DHA to $\Delta^4$-androstenedione. Microsomes were obtained from combined cell bands IV and V isolated from rat preputial gland. Incubation medium consisted of 0.1 M citrate-phosphate buffer (●) or 0.1 M Tris (○) of the designated pH, containing 5 μmol of NAD and [1,2-$H^3$]DHA ($10^6$ dpm) and 100 nmol of nonradioactive DHA. The pH was read immediately before the addition of the steroid. Optimum pH was approximately 8.5.

### *Time Course for the Formation of Androstenedione and Testosterone*

Fig 4 shows the time course of the formation of [$^3$H]$\Delta^4$-androstenedione and [$^3$H]testosterone when [$^3$H]DHA was incubated with the microsomal fraction of cells isolated from the preputial gland. Under the conditions as specified in the legend to Fig 4, 43% of the incubated [$^3$H]DHA was converted into $\Delta^4$-androstenedione in 20 min of incubation, while testosterone formation was not measurable until after 30 min of incubation. Based on these data, in further experiments for assaying 3β-HSD activity, the incubations were terminated at 20 min.

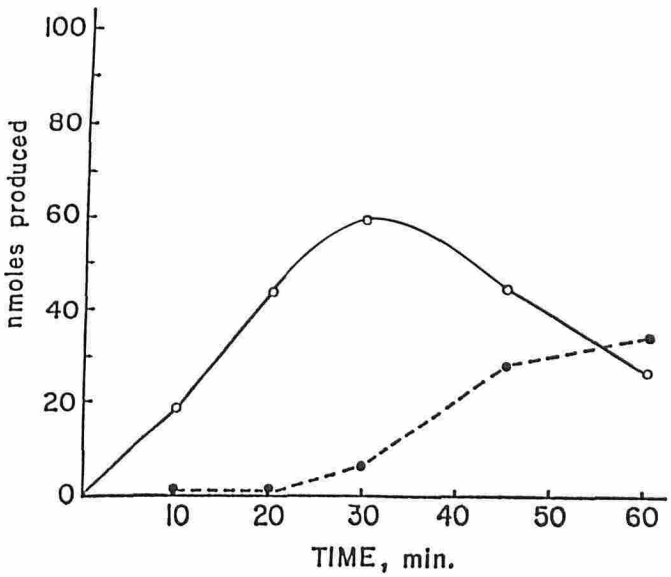


FIG 4. Time course of DHA conversion to $\Delta^4$-androstenedione (○) and testosterone (●). The incubation conditions were the same as described for Fig 3, except that the pH was held at 8.5, and time of incubation varied as indicated.

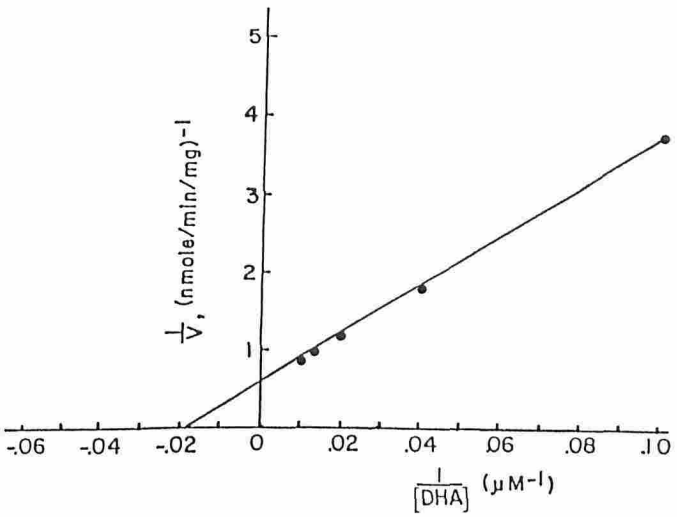


FIG 5. Lineweaver-Burk plot of the effect of DHA concentration of the rate of $\Delta^4$-androstenedione formation. The microsomal fraction was prepared from cells in bands IV and V, separated by Ficoll gradient centrifugation. The incubation mixture consisted of the microsomal fraction (2 mg protein) [1,2-$^3$H]DHA ($10^6$ dpm) diluted with nonradioactive DHA (1–100 μM), and 5 μmol of NAD in 1 ml of 0.1 M citrate phosphate buffer, pH 8.5. The mixture was incubated for 20 min at 37°C. The apparent $K_m$ for DHA estimated was $5.5 \times 10^{-5}$M; the $V_{max}$ was 1.72 nmol/min/mg microsomal protein.

### *Estimation of $K_m$ for 3β-HSD*

Fig 5 shows a Lineweaver-Burk plot for the estimation of apparent $K_m$ for DHA of 3β-HSD in the microsomal fraction of cells in bands IV and V isolated from the preputial gland. The concentrations of DHA tested were in the range of 1–100 μM. The plot was constructed with the help of Univac computer, using the HYPER program of Henson et al [13]. A value of $5.5 \times 10^{-5}$ M was obtained for the $K_m$, and a value of 1.72 nmol/min/mg microsomal protein was obtained for $V_{max}$.

## DISCUSSION

The in vivo experiment on the uptake of [$^3$H]DHA after intracardial administration (Fig 1) demonstrated that the pre-

putial gland, an androgen-sensitive organ, accumulated DHA, while the diaphragm, which is a non-target organ for androgens, does not. The initial high concentration of [$^3$H]DHA in the diaphragm was apparently due to the high concentration of [$^3$H]DHA in the arterial blood, which reached the diaphragm earlier than the preputial gland, after intracardial injection. The high $^3$H content in diaphragm, however, was rapidly diluted by subsequent circulation, while the content of $^3$H in preputial gland continued to rise, due to retention of [$^3$H]DHA and its metabolites. The data in Fig 2 show that [$^3$H]DHA is readily metabolized in the preputial gland. The metabolites identified were $\Delta^4$-androstenedione, testosterone, androstane-3$\alpha$,17$\beta$-diol, and DHT. The accumulation of HDA and its metabolites in the preputial gland was not due merely to their solubility in lipids of the gland, as cytosolic proteins of preputial gland which bind androstenedione, testosterone, and DHT have been demonstrated [14].

Formation of DHA metabolites was not detected in the diaphragm. The metabolites of DHA in the preputial gland formed in vivo were the same as those formed in vitro by isolated cells of the gland [11], except that in addition, a small amount of $^3$H was found in androstane-3$\alpha$,17$\beta$-diol. Formation of these metabolites indicates the presence of 17$\beta$-hydroxysteroid dehydrogenase, testosterone 5$\alpha$-reductase, and 3$\alpha$-hydroxy-steroid dehydrogenase in the gland, in addition to 3$\beta$-HSD. These findings imply that the androgenic effect of DHA may be mediated by the formation of DHT in the gland. Whether the other metabolites per se, can have specific hormonal influence on the gland without being converted into DHT is not known.

In our previous study, we observed a pronounced decrease of DHT content in the preputial gland after adrenalectomy of both male and female rats [11]. Taken together, these findings support the view that the adrenal cortex, by supplying DHA, serves as an important source of androgen for the preputial gland.

The first step in the metabolism of DHA is its conversion of $\Delta^4$-androstenedione, requiring a $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase and a $\Delta^{4\text{-}5}$-isomerase, both of which are membrane-bound in mammalian tissues. In the present study as well as in previous studies of human placenta [15,16], rat adrenal [17, 18], and rat ovary [19], the dehydrogenase activity was found to be rate-limiting, as no $\Delta^5$-androstenedione could be detected as a result of incubation of DHA with the enzyme preparations.

Table I indicates that the activity of 3$\beta$-HSD in the preputial gland was largely associated with the microsomal fraction (45%), while the mitochondrial fraction contained relatively less activity (24.5%). The specific activity of the enzyme in the microsomal fraction (25.7 nmol/h/mg protein) was nearly 4 times that in the mitochondrial fraction. This pattern of distribution is different from that found in human placenta, in which the specific activity of the enzyme was comparable in the mitochondrial and the microsomes fractions [15,16]. The pattern of distribution in rat adrenal [17,18] and rat ovary [19] was reportedly similar to that in the placenta.

Like other hydroxysteroid dehydrogenases, 3$\beta$-HSD in the preputial gland utilizes preferentially NAD. The high pH optimum of 8.5 of the preputial gland enzyme may be compared to that found in placenta (pH 10–10.5) [16]. The apparent $K_m$ of 3$\beta$-HSD in the preputial gland for DHA ($5.5 \times 10^{-5}$ M) was similar to that found for the human placenta enzyme ($5 \times 10^{-5}$ M) [15] and rat adrenal enzyme ($3.5 \times 10^{-5}$ M) [18].

The present study presents evidence that DHA secreted by the adrenal cortex may have an important adrogenic influence in regulating the activities of the preputial gland via its conversion to DHT and other metabolites. However, caution must be exercised in extrapolating these findings to the human sebaceous gland, as there are vast morphologic, phylogenetic, and functional differences. Nonetheless, the findings on the preputial gland are useful for the illustration of the action of the adrenal androgen in a target organ with sebaceous characteristics.

## REFERENCES


1. Marynick SP, Chakmakjian ZH, McCaffree DL, Herndon JH Jr: Androgen excess in cystic acne. N Engl J Med 308:981–986, 1983
2. Pochi PE, Strauss JS, Meseon H: The role of adrenocortical steroids in the control of human sebaceous gland activity. J Invest Dermatol 41:391–399, 1963
3. Pochi PE, Strauss JS: Sebaceous gland response in man to the administration of testosterone, $\Delta^4$-androstenedione and dehydroisoandrosterone. J Invest Dermatol 53:32–36, 1969
4. Drucker WD, Blumberg JM, Gandy HM, David RR, Verde AL: Biological activity of dehydroepiandrosterone sulfate in man. J Clin Endrocrinol Metab 35:45–54, 1972
5. Cameron EHD, Baillie AH, Grant JK, Milnes JA, Thomas J: Transformation in vitro of [$^3$H] testosterone by skin from men. J Endocrinol 35:XIX, 1966
6. Hay JB, Hodgins MB: Metabolism of androgens in vitro by human facial and axillary skin. J Endocrinol 59:475–486, 1973
7. Oertel GW, Treiber L: Metabolism and excretion of $C_{19}$- and $C_{18}$-steroids by human skin. Eur J Biochem 7:234–238, 1969
8. Simpson NB, Cunliffe WJ, Hodgins MB: The relationship between the in vitro activity of 3$\beta$-hydroxysteroid dehydrogenase $\Delta^{4\text{-}5}$-isomerase in human sebaceous glands and their secretory activity in vivo. J Invest Dermatol 81:139–144, 1983
9. Gomez EC, Hsia SL: Studies on cutaneous metabolism of testosterone-4-$^{14}$C and $\Delta^4$-androstene-3,17-dione-4-$^{14}$C in human skin. Biochemistry 7:24–32, 1968
10. Huggins C, Parson FM, Jensen EV: Promotion of growth of preputial gland by steroids and the pituitary growth hormone. Endocrinology 57:25–32, 1955
11. Hsia SL, Sawaya ME, Voigt W: Transformation of dehydroepiandrosterone into dihydrotestosterone by isolated cells from rat preputial gland. J Steroid Biochem 19:599–603, 1983
12. Potter JER, Prutkin L, Wheatley VR: Sebaceous gland differentiation. I. Separation, morphology and lipogenesis of isolated cells from mouse preputial gland tumor. J Invest Dermatol 72:120–127, 1979
13. Hensen R, Ling R, Havir E: A computer program for fitting data to the Michaelis-Menten equation. Biochem Biophys Res Commun 29:194–197, 1967
14. Voigt W, Sawaya ME, Hsia SL: Mechanism of action of dehydroepiandrosenrone in the androgenic stimulation of sebaceous glands. Abstract presented at the International Symposium on Regulation of Androgen Action, Montreal, Quebec, Canada, June 28–30, 1984
15. Ferre F, Breuiller M, Cedand L, Duchesne MJL, Saintot M, Descomps B, Crasts DePault A: Human placental $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase activity ($\Delta^5$-3 HSDH): intracellular distribution, kinetic properties, retroinhibition and influence of membrane delipidation. Steroids 26:551–570, 1975
16. Koide SS, Torres MT: Distribution of 3$\beta$-hydroxysteroid dehydrogenase and $\Delta^5$-3-oxosteroid isomerase in homogenate fractions of human term placenta. Biochim Biophys Acta 105:115–120, 1965
17. McCune RW, Roberts S, Young PL: Competitive inhibition of adrenal $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase and $\Delta^5$-3-ketosteroid isomerase activities by adenosine 3′,5′-monophosphate. J Biol Chem 245:3859–3867, 1970
18. Basch RS, Finegold MJ: 3$\beta$-Hydroxysteroid dehydrogenase activity in the mitochondria of rat adrenal homogenates. Biochem J 125:983–989, 1971
19. Sulimovici S, Boyd GS: The $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase of rat ovarian tissue: the effect of adenosine 3′,5′-cyclic-monophosphoric acid. Eur J Biochem 7:549, 1969